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MASS SPECTRAL INVESTIGATIONS ON TOXINS

VII. DETECTION AND ACCURATE QUANTITATION OF PICOGRAM QUANTITIES OF MACROCYCLIC TRICHOTHECENES IN BRAZILIAN PLANT SAMPLES BY DIRECT CHEMICAL IONIZATION-MASS SPECTROMETER/ MASS SPECTROMETER TECHNIQUES

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Aberdeen Proving Ground, Maryland 21010-5423

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Roridins and baccharinoids are macrocyclic trichothecenes. They formed $(M+NH_4)^+$ adducts effectively with ammonia under chemical ionization conditions. The daughter spectra of these adducts were characteristic of the macrocyclic ester bridges and indicate several common neutral losses. Experiments designed for the simultaneous detection of these macrocyclic trichothecenes by sequentially monitoring their characteristic daughter ions and the corresponding parent spectra were found to be applicable for the analysis of Brazilian Baccharis plants. The compounds detected in these samples were accurately quantified using a synthetically modified macrocyclic trichothecene, 8-ketoverrucarin A, as the internal standard. The minimum detectable limits of roridins and baccharinoids under various experimental conditions used for their detection and quantification were measured to be 10-100 pg.

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16. Supplementary Notation (Continued)

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Direct chemical ionization-mass spectrometry/mass spectrometry

PREFACE

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1. INTRODUCTION

Macrocyclic trichothecenes are polar, toxic molecules originating from several species of fungi such as myrothecium, stachybotrys, etc.[1-3] These molecules are the most toxic of all known trichothecenes.[4-6] The loss of livestock due to animals ingesting feed infested with these fungi has been recognized for decades.[1-11] Recently, several human health problems have also been attributed to these macrocyclic trichothecenes.[11-13*] These toxic compounds have been detected and isolated from environmental and agricultural samples and fermentation broths. However, few toxic macrocyclic trichothecenes (roridins) along with some biologically active (chemotherapeutic) compounds [baccharinoids (baccharinols)] have been isolated from haccharis megapotamica plants in Brazil.[4,14] The presence of the toxic roridins in the baccharis plants was originally recognized when cattle feeding on the plants in Brazil died.[4] Extensive investigations by Jarvis and others have led to the isolation and characterization of several toxic macrocyclic trichothecenes, along with a few beneficial ones, from these plants.[4]

To prevent economic loss and health hazards, the development of rapid, specific, and sensitive methods of analyzing macrocyclic trichothecenes has become essential for detecting toxic roridins in various types of samples. This development is also required for detecting toxic roridins in purified samples of baccharinoids. However, the available methodology for the analysis of any of these macrocyclic trichothecenes is very limited. Analysis of these trichothecenes by gas chromatography/mass spectrometry (GC/MS) is impossible due to the presence of polar, labile ester bridges in the molecules.[5-16**]

We have developed a direct, rapid, selective, and sensitive direct chemical ionization (DCI)-MS/MS method to detect Baccharinoid-4 (B4) and Baccharinoid-5 (B5) in the presence of four frequently observed toxic roridins.[17] The mixture of roridins and baccharinoids was subjected to chemical ionization (CI) in the presence of ammonia followed by the sequential collisionally activated dissociation (CAD) of the specific ammonium adducts using argon. Experimental conditions for simultaneously analyzing these molecules by detecting their specific daughter ions and associated parent ions during a single analysis were developed. A synthetically modified macrocyclic trichothecene,[14] 8-ketoverrucarin A (KVA), was used as the internal standard for accurately quantifying detected compounds, and this procedure was applied in analyzing several Brazilian baccharis plant samples.

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*Some of the information was obtained from a personal communication with W.A. Croft, B.B. Jarvis, and C.S. Yatawasa, 1985.

**Part of the information provided was obtained from unpublished data by T. Krishnamurthy and E.W. Sarver of CRDEC and B.B. Jarvis and S.L. Greene of the University of Maryland, 1985.

2. EXPERIMENTAL PROCEDURE

All CI and CAD experiments were conducted using the commercially available Finnigan-MAT TSQ triple quadrupole MS. The roridins and baccharinoids standards and baccharis plant extracts were generously donated by Professor Bruce B. Jarvis of the University of Maryland (College Park, MD). Standard solutions were prepared using glass-distilled methanol and stored at 0 °C in reacti-vials fitted with mininert valves (Supelco Incorporated, Supelco Park, PA).

Analysis of Standards and Baccharis Plant Samples.

One microliter of standard mixture (solution) was loaded on the direct exposure probe (DEP) filament and evaporated. The DEP was introduced into the MS, and the sample was subjected to CI using ammonia at 100 °C with a source pressure of 0.45 torr. The probe was heated from 140 °C to 270 °C at 100 °C/sec. The ammonium adducts were dissociated in the collision chamber using argon at a pressure of 2-2.3 mTorr and 1 mTorr for the daughter and parent spectra experiments, respectively. The collision energies were maintained at -15 eV. The baccharis plant samples and the internal standard (KVA) were analyzed under identical conditions.

3. RESULTS AND DISCUSSION

Macrocyclic trichothecenes are naturally occurring di- and triesters of unsubstituted and substituted verrucarols.[1-4] The diesters are termed as roridins, satratoxins, and baccharinoids, depending upon the structural features of the verrucarol and ester moieties.[4] The triesters are verrucarins.[4] These trichothecenes are all acutely toxic, polar compounds isolated from various fungal sources.[1-3] However, some of the macrocyclic trichothecenes (baccharinoids) with substituents at the A ring (Figure 1) possess strong antileukemic properties.[4] Along with toxic roridins, several baccharinoids have been isolated from Brazilian baccharis plants.[4] The latter molecules were transformed into the beneficial, biologically active baccharinoids under chemical or microbial conditions.[4,5] Under the same conditions, similar results were observed with the verrucarins.[4,5]

The economic loss and human and animal health problems caused by the macrocyclic trichothecenes have been recognized and documented.[1-13*] To detect the trichothecenes and prevent any losses and/or disasters, methods are required for specifically detecting and accurately quantifying these toxic molecules in environmental and agricultural samples. However, such analytical methods for macrocyclic trichothecenes were virtually unknown until recently.[15,16**] Direct GC/MS analysis of the compounds or their derivatives is not possible due to the insufficient volatility of the molecules and the presence of labile ester bridges. A GC/negative ion chemical ionization (NICI)-MS method based

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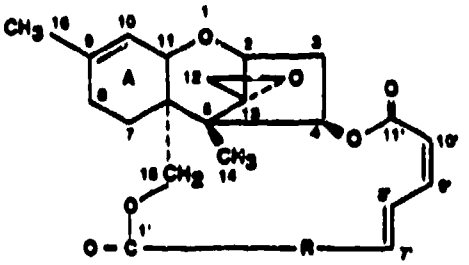
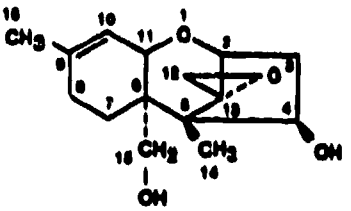




VERRUCAROL

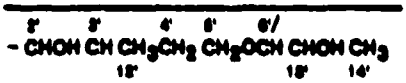
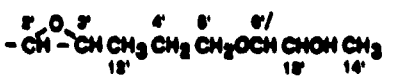
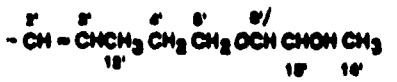
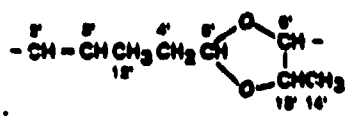
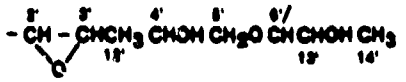
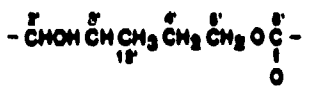
COMPOUND	R	OTHER
RORIDIN A		—
RORIDIN D		—
RORIDIN E		—
RORIDIN H		—
BACCHARINOL-4		8β-OH
BACCHARINOL-8		8,10-EPOXY
8-KETOVERRUCARIN A (KVA)		8-KETO

Figure 1. Macrocyclic Trichothecenes

on the alkaline hydrolysis of the molecules, followed by derivation of the hydrolysate (verrucarols) and GC/MS analysis of the derivatives has been developed.* Despite being useful in simultaneously detecting several related macrocyclic trichothecenes (verrucarols), this method identifies the verrucarol moieties but not the ester bridges. Earlier, we reported that under CI (methane) conditions, the macrocyclic trichothecenes formed negatively charged molecular (M^-) ions more efficiently than positively charged protonated molecules.[15] Under CAD conditions, the M^- ions underwent fragmentation characteristic of the ester bridges; hence, these ions are useful in identifying the molecules.[15] Satratoxins produced M^- ions very efficiently despite the nature of the CI reagent gases.[16] The protonated molecules of satratoxins formed under these conditions were insufficient to conduct CAD experiments with the required high sensitivity.[16] Thus, a direct, negative DCI-MS/MS method for analyzing satratoxins was developed and applied in analyzing Stachybotris atra fermentation samples.[16]

In this report, we provide the results of an extensive investigation with respect to the optimum conditions for ionizing the macrocyclic trichothecenes, the CAD of their adducts, and the simultaneous detection and quantification of the macrocyclic trichothecenes.

Roridins and baccharinoids produced M^- ions preferably over the positively charged protonated molecules in the presence of all common CI reagent gases except ammonia.[17] However, these molecules produced both M^- and $(M+NH_4)^+$ ions with almost equal efficiency, the latter formed in slight excess, when ammonia was used as the reagent gas. Optimum conditions for heating the sample in the source were determined by introducing the individual standards (50 ng) into the source via a DEP, heating the probe at 20 °C/sec in the presence of ammonia (CI), and recording the standards' positive ion chemical ionization (PICI) spectra. Careful analysis of the reconstructed ion chromatogram (RIC) and spectral data indicated the ideal temperature range in which the ionization rate of the molecules exceeded their thermal decomposition rate by several folds. Then, for increased sensitivity, each standard was introduced and analyzed under the same conditions except this time the probe was heated rapidly at 100 °C/sec over the temperature range characteristic of each molecule. The initial and final temperatures of the probe and the rate of heating were adjusted to produce narrow, sharp RIC peaks for increased sensitivity of detection with negligible thermal decomposition of the analytes. The optimum temperature ranges thus determined for individual molecules are listed in Table 1.

The molecular ion-ammonium adducts produced were subjected to CAD in the presence of argon. The total scan of daughter spectra of individual molecules (50 ng) were recorded at a constant collision gas pressure of 2.3 mTorr, varying the collision energy stepwise. The optimum collision energy for the compound was determined based on the relative abundances of the $(M+NH_4)^+$, $(M+H)^+$, and the most characteristic daughter ions. The optimum collision gas pressures for individual molecules were determined similarly. The optimum CAD conditions for obtaining the daughter spectra $(M+NH_4)^+$ and M^- ions

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of all compounds are listed in Table 2. The positive CAD spectra of roridins and B4 and B5 are shown in Figures 2-7. The corresponding CAD ions from the negative daughter spectra are listed in Table 2.

Table 1. Optimum CI and CAD Conditions

COMPOUND	DEP TEMPERATURE* (°C) MINIMUM	DEP TEMPERATURE* (°C) MAXIMUM	COLLISION GAS PRESSURE (mtorr) $(M+NH_4)^+$	COLLISION GAS PRESSURE (mtorr) M^-	COLLISION ENERGY (ev) $(M+NH_4)^+$	COLLISION ENERGY (ev) M^-
RORIDIN A	135	230	2.30	2.0	-15.0	18.0
RORIDIN D	150	220	2.30	2.3	-14.0	24.0
RORIDIN E	140	200	2.30	2.3	-14.0	19.0
RORIDIN H	140	280	2.30	2.3	-14.0	18.0
B4	175	270	2.30	2.3	-14.0	21.0
B5	175	270	2.30	2.3	-14.0	20.0

*Rate of heating: 100 °C/sec

Table 2. The MS/MS Spectra (Negative Ions) of Roridins and Baccharinoids

COMPOUND	M · W	m/z (RELATIVE ABUNDANCE)
RORIDIN A	532	402 (15.6), 401 (100.0), 153 (19.6), 145 (69.8)
RORIDIN D	530	530 .7), 417 (1.3), 401 (100.0), 359 (2.3) 349 (1.0), 193 (2.5), 175 (1.1), 153 (7.1) 151 (1.2), 149 (2.5), 145 (3.1), 135 (13.1) 127 (1.2),
RORIDIN E	514	514 (14.9), 484 (7.0), 470 (2.1), 403 (71.4) 386 (3.4), 385 (17.2), 359 (100.0), 154 (6.2) 153 (20.3), 136 (10.7), 134 (3.8), 111 (8.1)
RORIDIN H	512	512 (1.1), 466 (1.4), 403 (5.8), 401 (2.2) 387 (10.1), 385 (6.5), 359 (24.3), 219 (1.3) 175 (1.8) 153 (4.0), 137 (11.1), 125 (100.0) 109 (25.8),
B4	562	562 (3.6), 501 (10.1) 417 (100.0), 375 (2.4) 365 (29.9), 161 (7.4), 153 (19.9), 143 (12.8) 141 (1.2), 135 (12.9), 125 (12.2),
B5	562	562 (2.5), 501 (6.0), 417 (100.0), 375 (1.0) 365 (17.6), 279 (1.5), 161 (1.5), 153 (9.0) 135 (4.7), 125 (1.8),

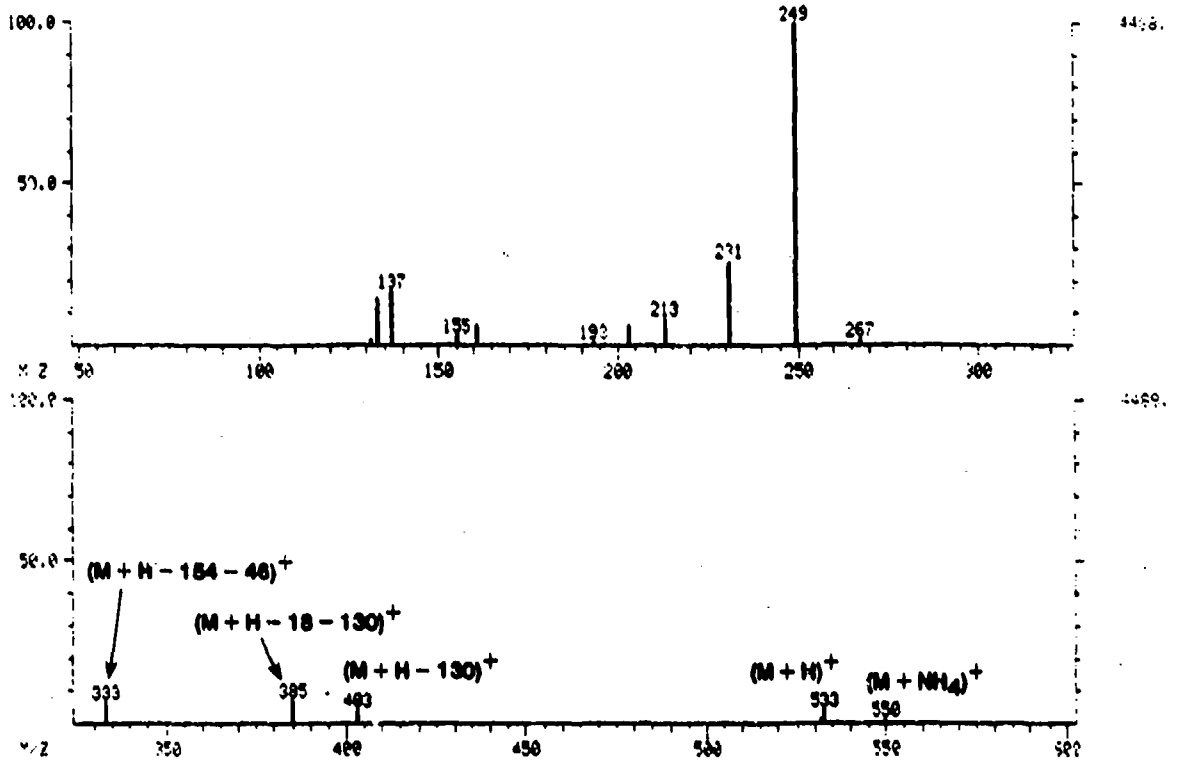


Figure 2. Daughter Spectrum of Roridin A - Ammonium Adduct (m/z 550)

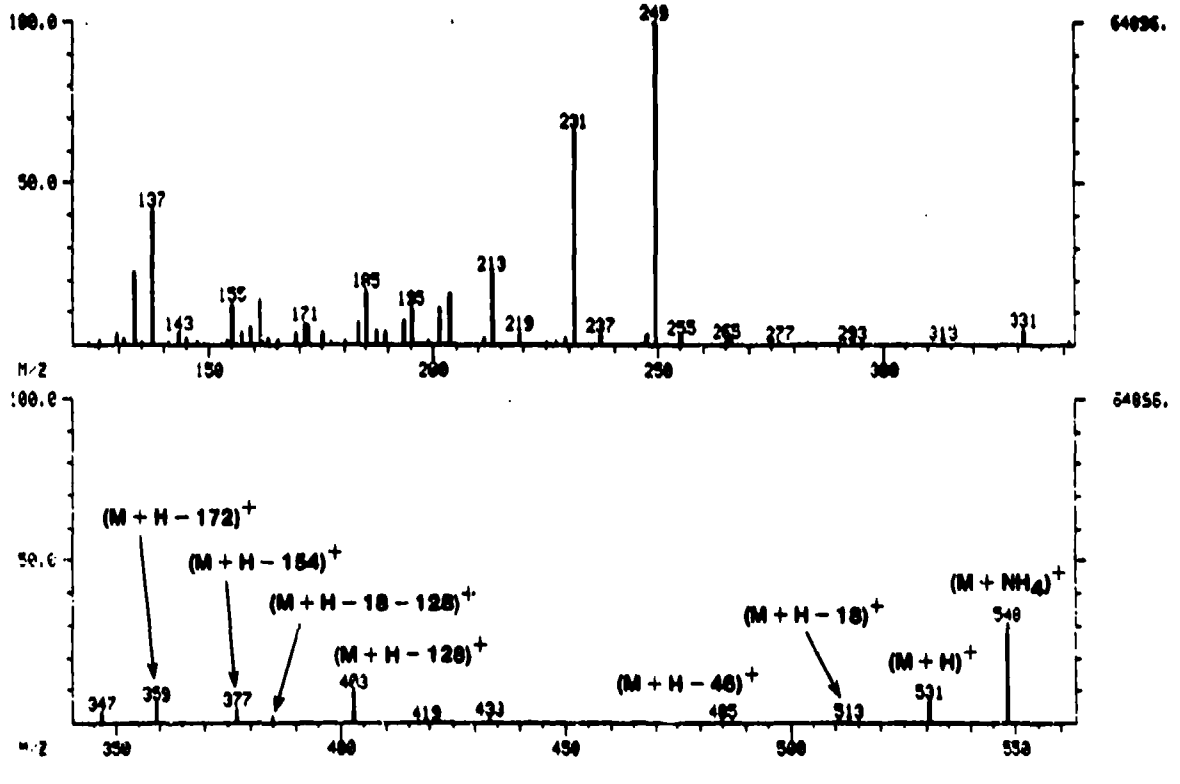


Figure 3. Daughter Spectrum of Roridin D - Ammonium Adduct (m/z 548)

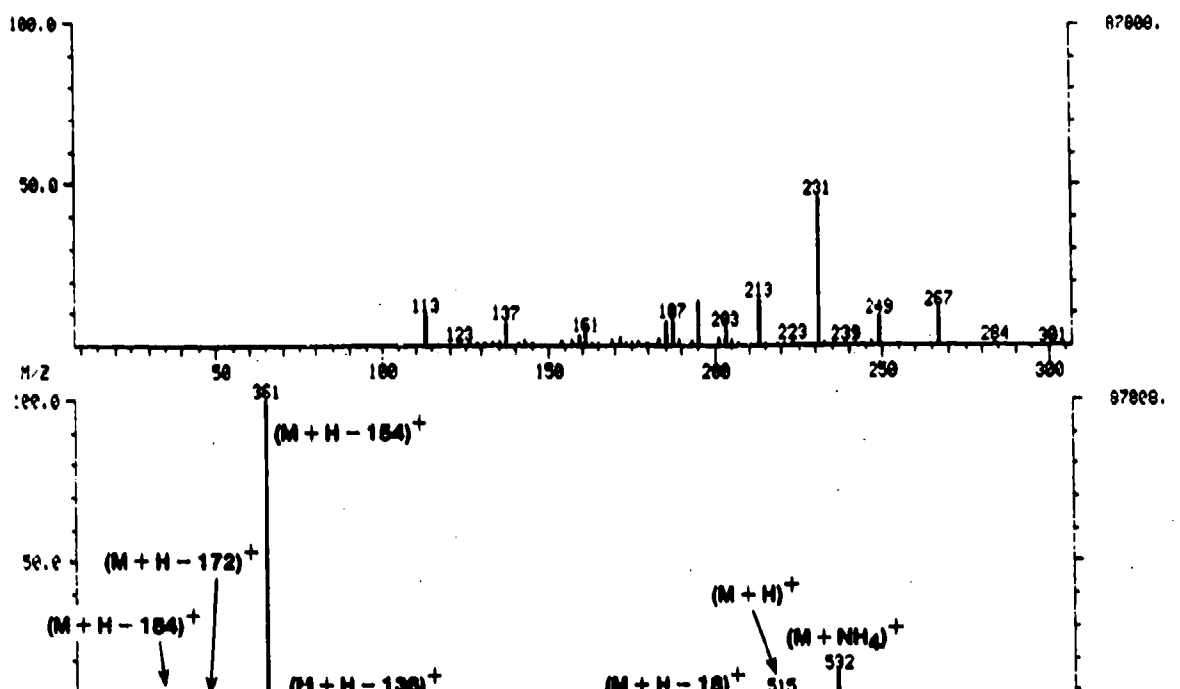


Figure 4. Daughter Spectrum of Roridin E - Ammonium Adduct (m/z 532)

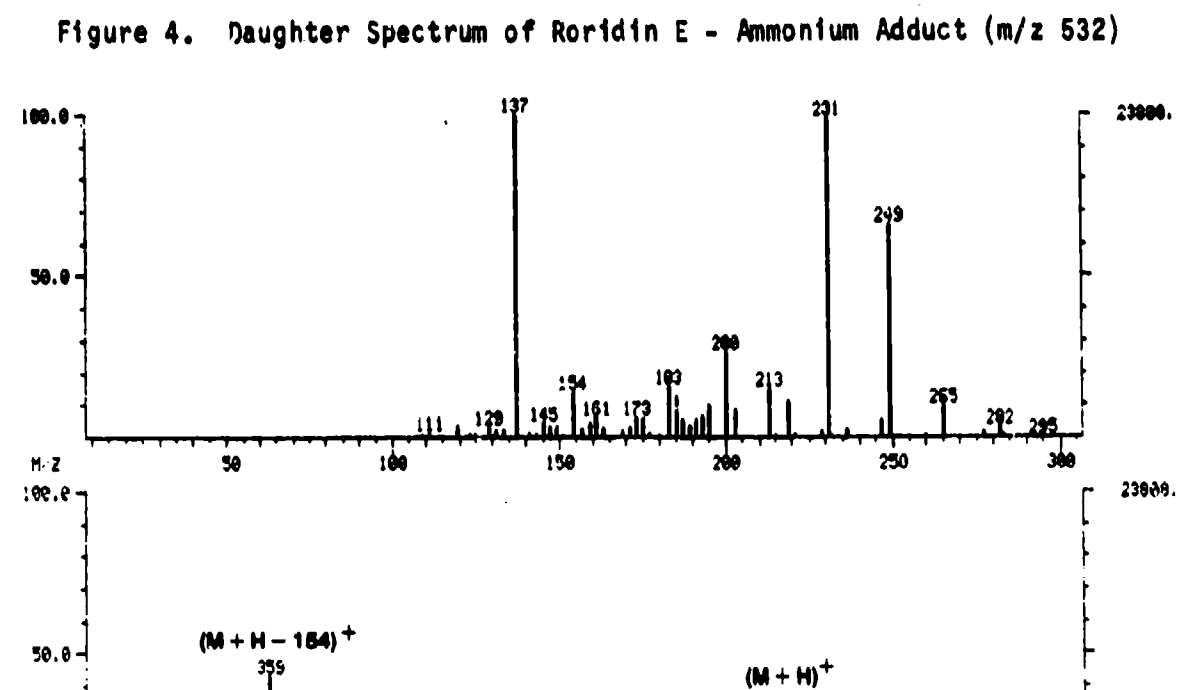


Figure 5. Daughter Spectrum of Roridin H - Ammonium Adduct (m/z 530)

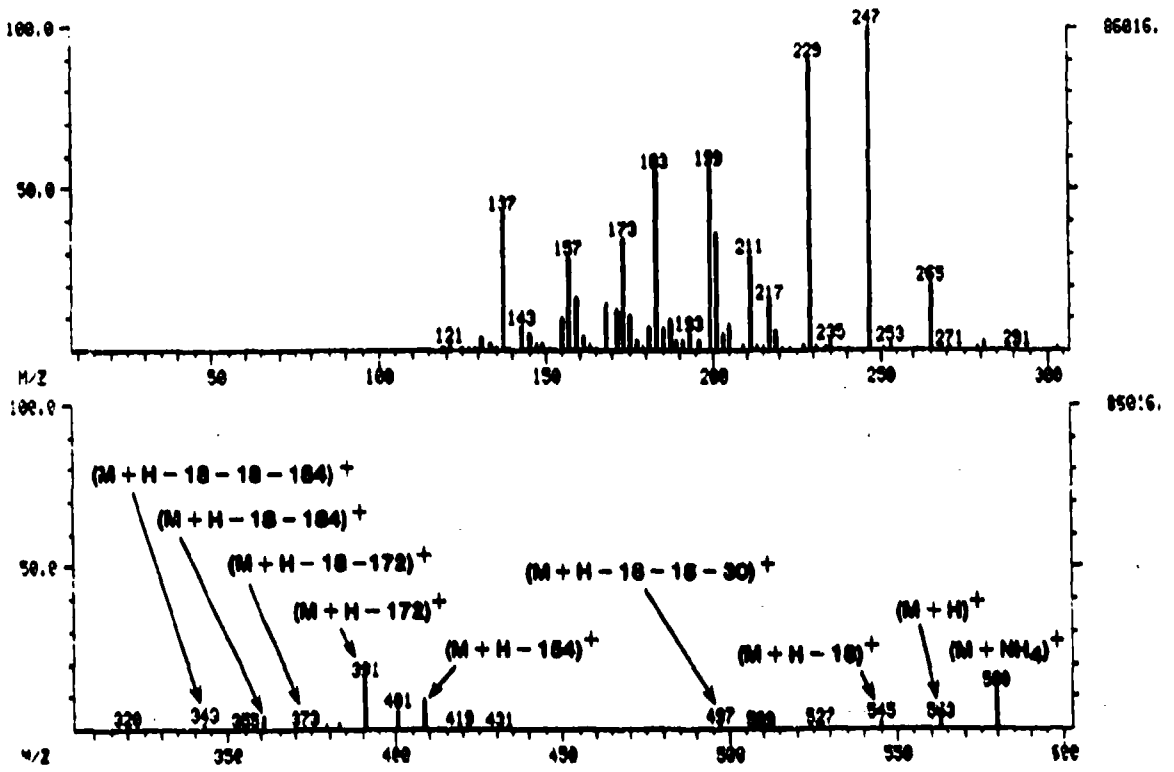


Figure 6. Daughter Spectrum of B4 - Ammonium Adduct (m/z 580)

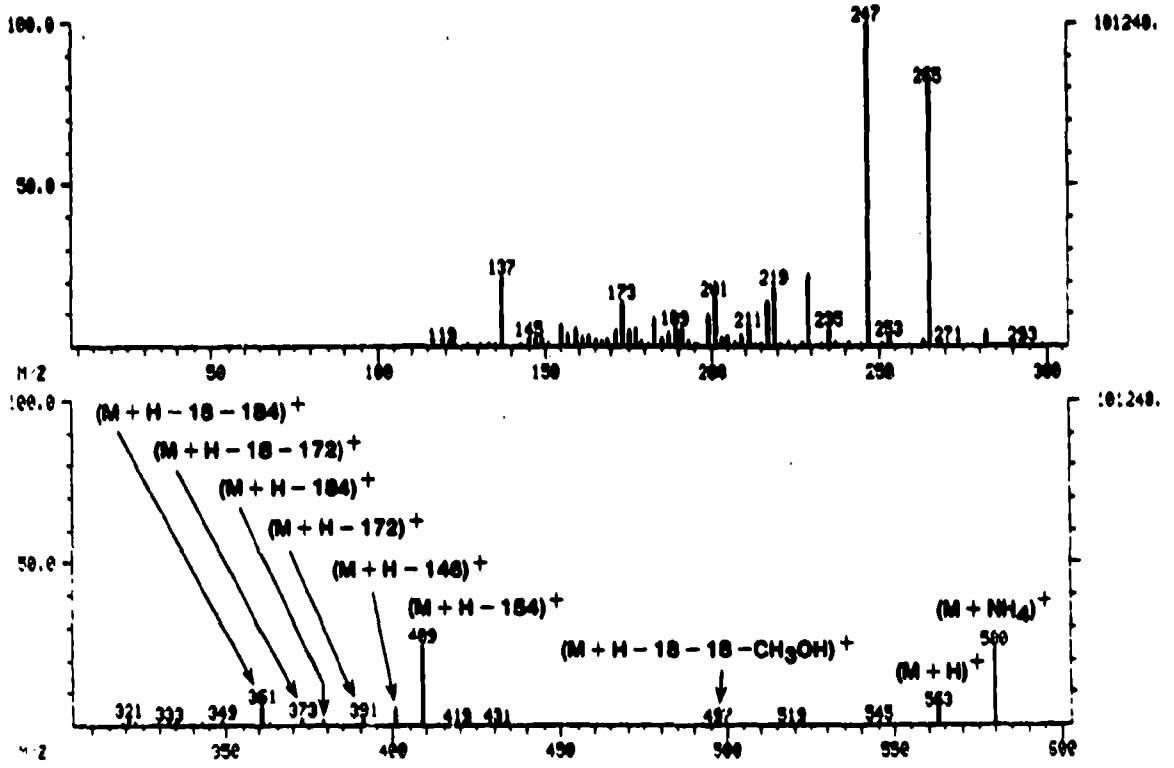


Figure 7. Daughter Spectrum of B5 - Ammonium Adduct (m/z 580)

The fragmentation mode of M^- ions of some of the macrocyclic trichothecenes under CAD conditions has been described in detail by Krishnamurthy and Sarver.[15] The CAD spectra of the positively charged ammonium adducts and M^- ions of four toxic roridins and B4 and B5 were characteristic of the ester bridges. The following pathways of the fragmentation of molecular ion-ammonium adducts under CAD conditions were proposed based on the mass per charge of the observed daughter ions as well as the masses of the neutral molecules ejected from the daughter ions during the processes. In all cases, under CAD conditions, the ammonium adduct lost the ammonia and all the daughter ions were formed from the protonated molecules. Most of the CAD ions seemed to come from the ester bridges of the protonated molecules. The observed cleavages during the process seemed to occur between C1'-O, C5'-O, O-C6', C11'-O, or C10'-C11' bonds initially. The bond cleavage between the C3' and C4' atoms led to the formation of ethanol. The proposed structures of some common daughter ions are listed in Figure 8. The suggested structures for the neutral losses are indicated in Schemes 1-4.

The proposed fragmentation pathways for the protonated molecules of Roridin A as well as the structures for the observed neutral losses are indicated in Scheme 1. Besides the loss of water and ethanol, neutral losses of 118, 130, 136, and 154 were also observed in Roridin A. The presence of m/z 131, m/z 137, and m/z 155 indicates these neutral losses. A cyclic lactone stucture was assigned to m/z 333 that was formed by the neutral loss of 154 followed by the loss of ethanol or vice versa. With m/z 331, ions comparable to m/z 333 were also noted for Roridins D, E, and H (Schemes 2-4). The proposed fragmentation pathways are clearly indicated in Scheme 1.

Neutral losses of 18, 46, and 128 (equivalent to m/z 130 observed in Roridin A) were observed in Roridin D. The daughter ion (m/z 359) was probably formed by the loss of the neutral molecule (172) or by the neutral loss of water and 154 from the protonated molecules. The presence of m/z 171 favored former transformation, i.e., neutral loss of 172. Similarly, m/z 347 was explained due to the neutral loss of 184 from the protonated molecules as well as to the presence of m/z 185. The latter two transformations are shown in Scheme 2.

Neutral losses observed in Roridin E were similar to those observed in Roridin D. The daughter ion (m/z 379) indicated in Scheme 3 seemed to form from the protonated molecule by the loss of water followed by the neutral loss of 136. The structure proposed for this lost neutral molecule is indicated in Scheme 3. The structures proposed for m/z 331 and m/z 113 are indicated in Scheme 3 and Figure 8, respectively.

For Roridin H, in addition to the usual neutral losses of 18, 46, 136, and 154, two additional losses with masses of 94 and 146 Daltons were also noted. The structures for the daughter ions arising from the latter losses are shown in Scheme 4. The proposed structure for the characteristic daughter ion (m/z 183) is shown in Figure 8. Similar neutral losses of 18, 146, 154, 172, and 184 were also noted in B4 and B5 as well. The structures for m/z 183 and m/z 199 are also indicated in Figure 8. The presence of m/z 183 clearly indicates the neutral loss of 184 in B4 and B5 and not the loss of 28 followed by the loss of 156, etc. The relative intensities of these ions and m/z 201 and m/z 219 were different in B4 and B5 and should arise from ring A.

The neutral loss of 154, 172, and 184 could be used to detect the roridins and baccharinoids by scanning the tandem mass spectrometer in the neutral loss mode.

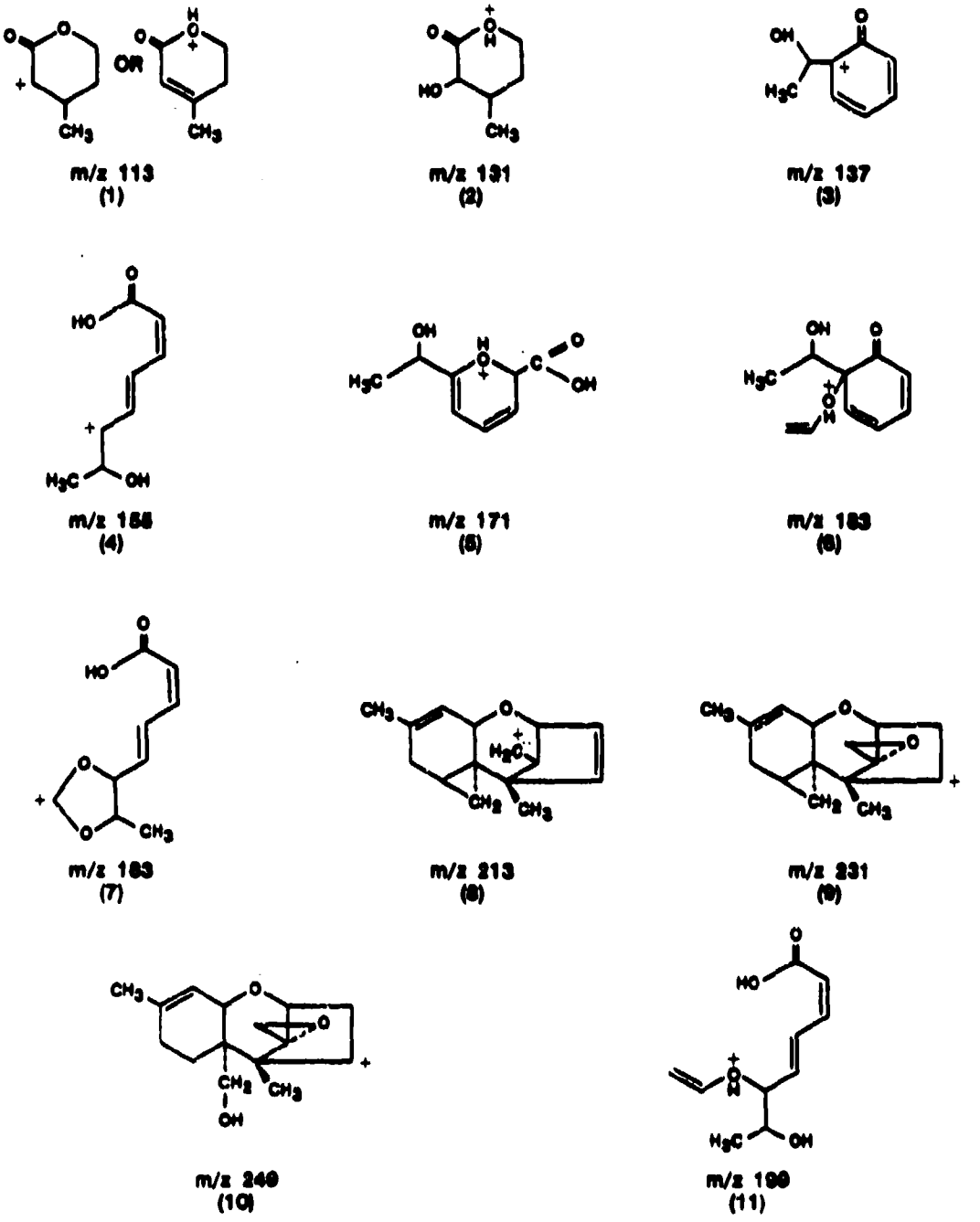
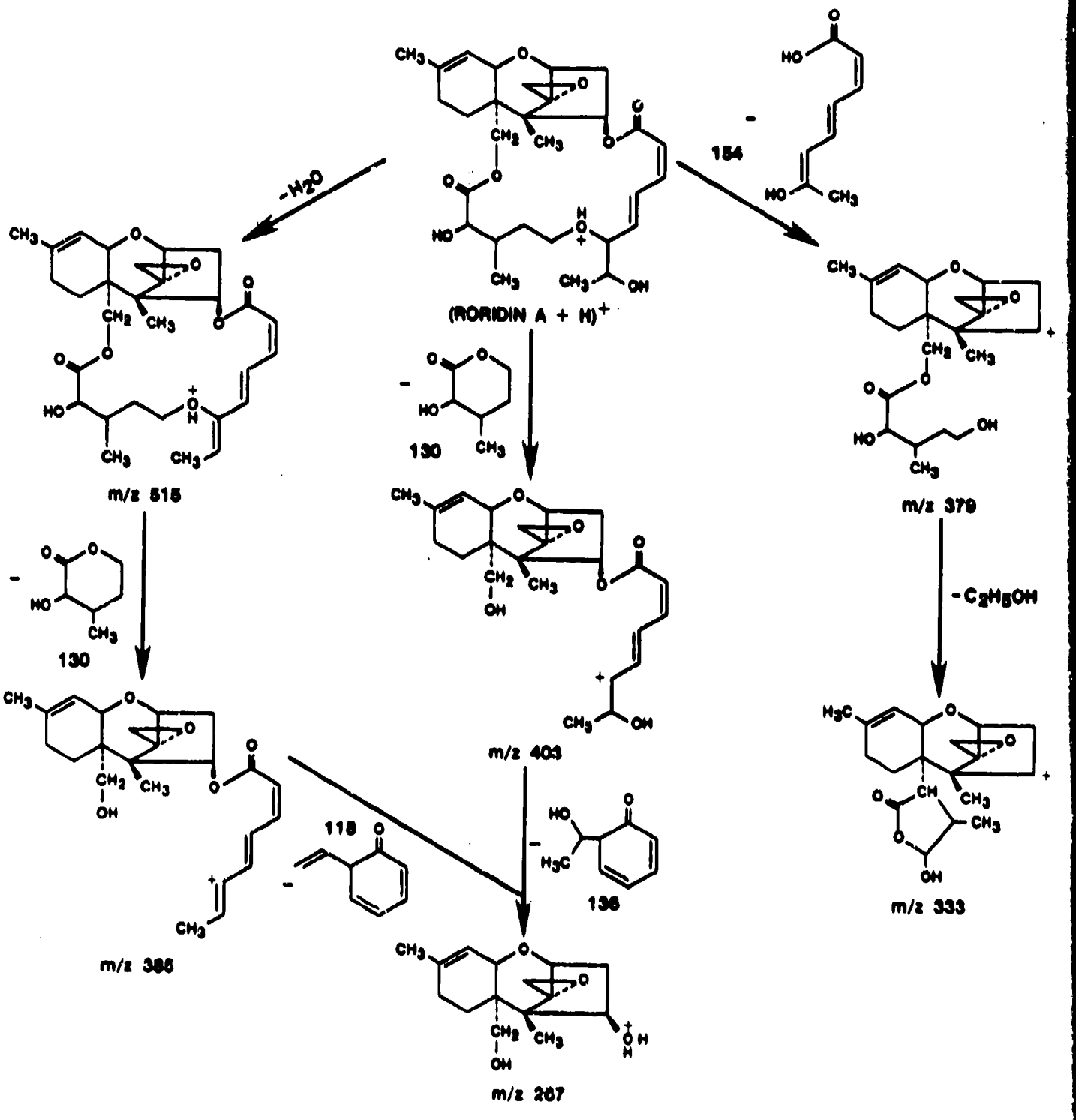
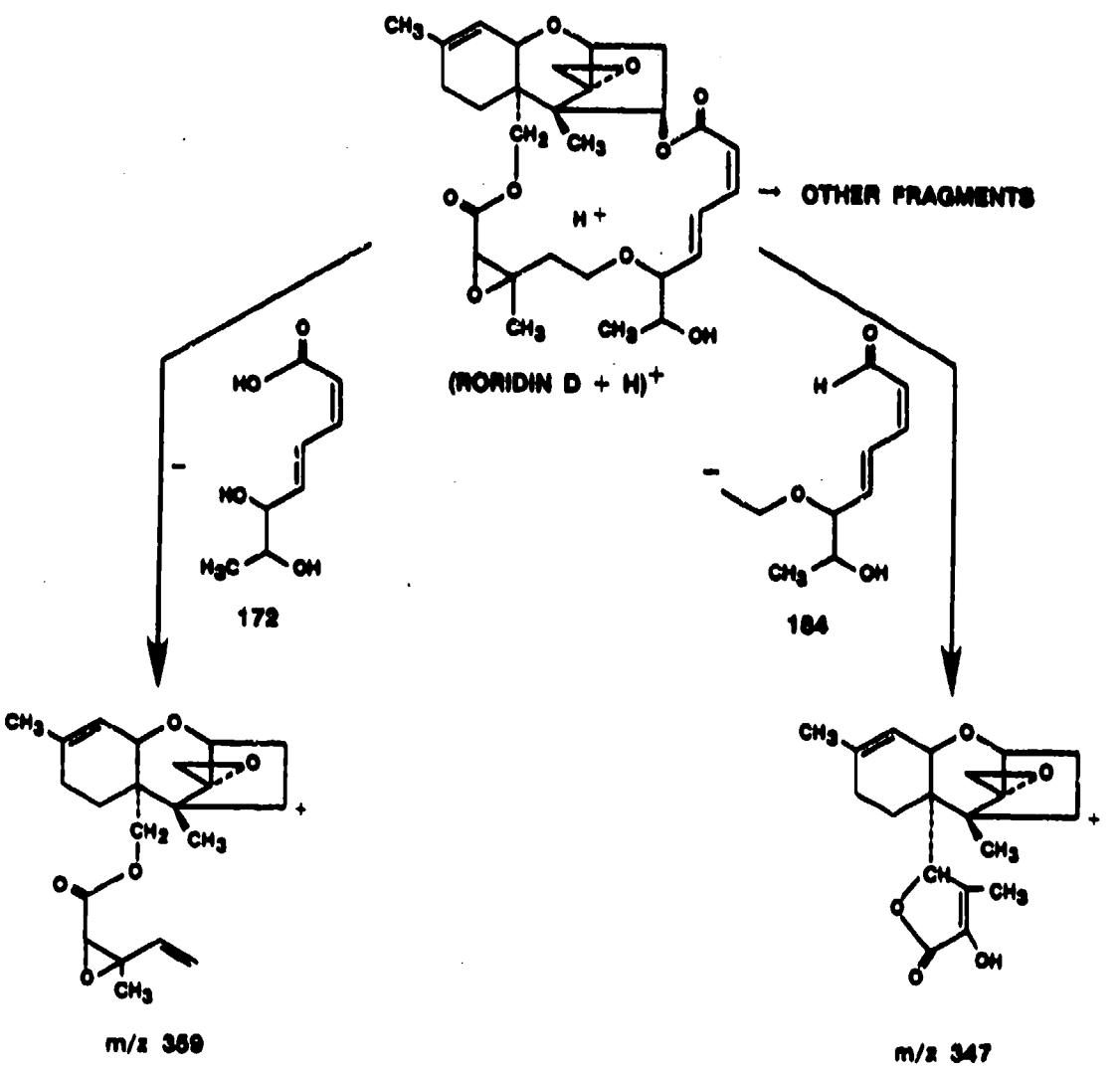


Figure 8. Proposed Structures for Some Common Daughter Ions

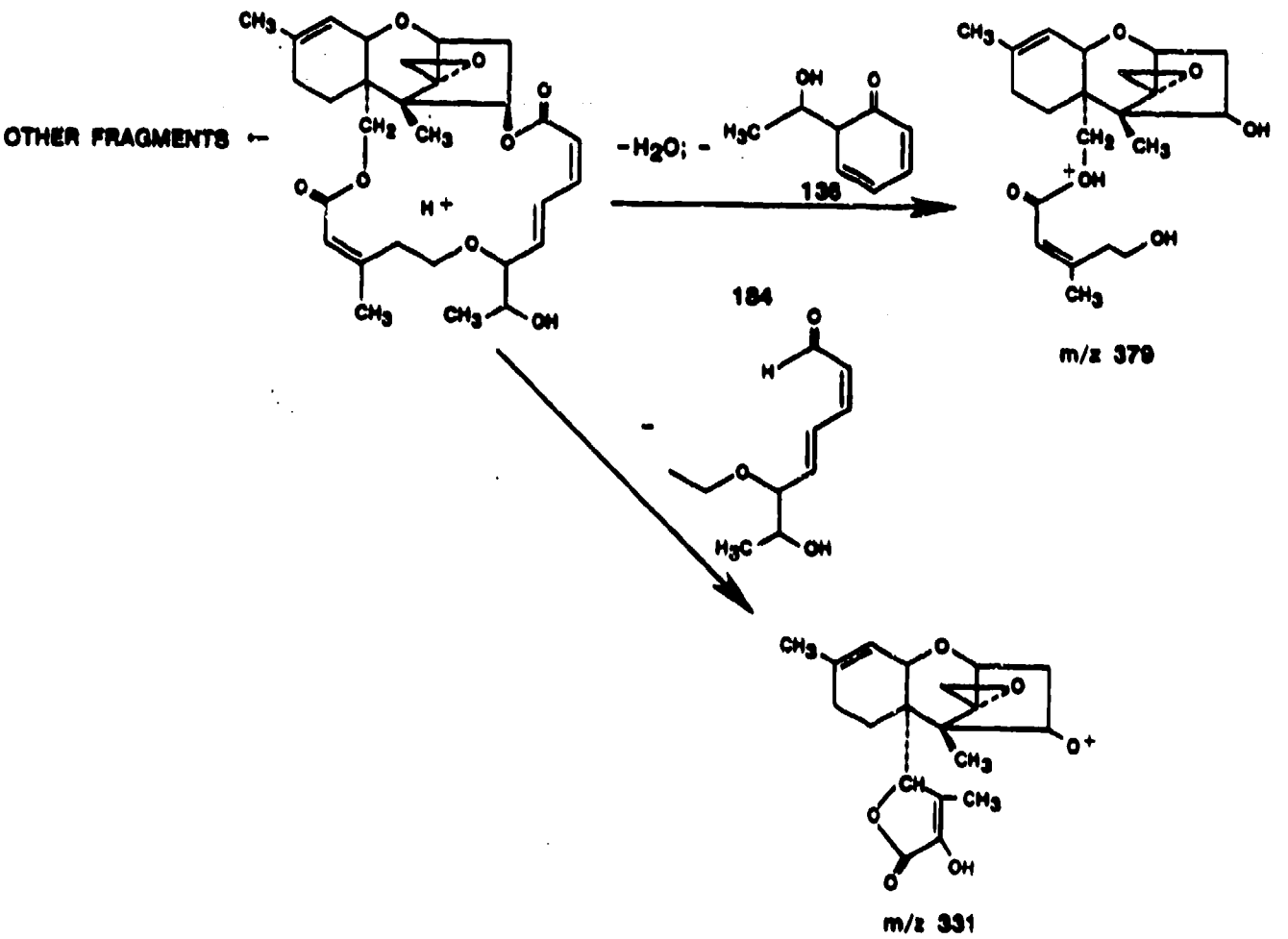
Scheme 1


(RORIDIN A + H)+
-H2O
154
130
m/z 515
m/z 379
-C2H5OH
130
m/z 403
118
136
m/z 385
m/z 333
m/z 267

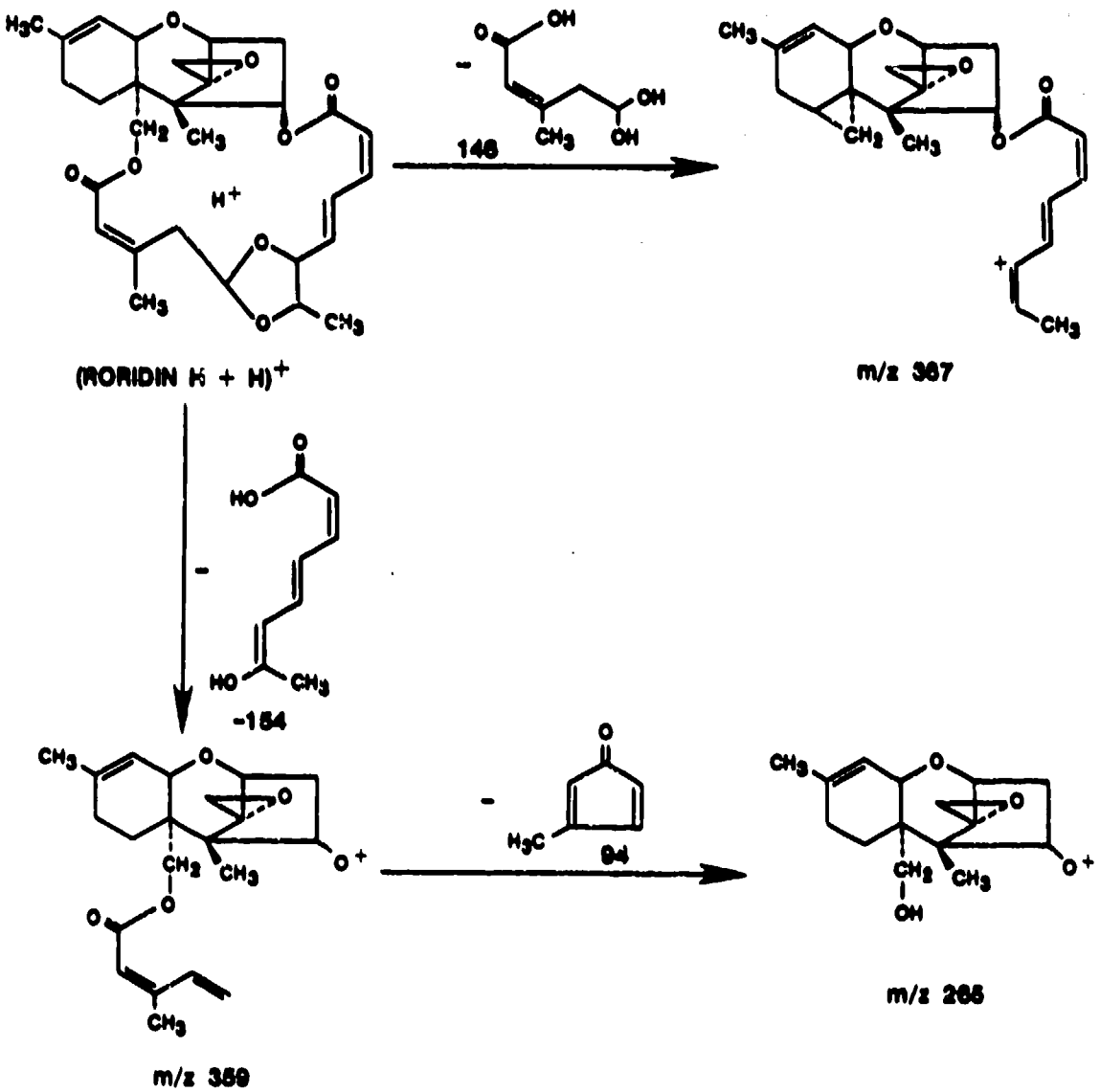
Scheme 2



Scheme 3



Scheme 4



Three of the most intense daughter ions were chosen from positive and negative ion CAD spectra of samples of roridins and baccharinoids. Experiments RS and RB (Table 3) were designed and used for the simultaneous identification of roridins and baccharinoids, respectively, in some baccharis plant sample extracts. Initially, the standard mixture (1 µl) containing 25 ng of standards was introduced into the source via a direct insertion probe and subjected to CI in the presence of ammonia. The ammonium adducts of each molecule were introduced sequentially into the collision chamber and dissociated with argon. An experiment (program) (Table 3) was designed to monitor sequentially the three characteristic daughter ions for each molecule: 1 µl of the plant extracts was also analyzed. The whole process was computer controlled, and the measured relative abundances are listed in Table 4.

Table 3. Screening Conditions for Roridins and Baccharinoids

EXPERIMENT	COMPOUND	Q_1	CAD CONDITIONS C.G.P (mtorr)	CAD CONDITIONS C.E (ev)	Q_3
RS	RORIDIN A	550	2.3	−15	137, 231, 249
	RORIDIN D	548	2.3	−14	137, 231, 249
	RORIDIN E	532	2.3	−14	231, 267, 361
RB	RORIDIN H	530	2.3	−14	137, 231, 359
	BACCHARINOL-4	580	2.3	−14	137, 157, 247
	BACCHARINOL-5	580	2.3	−14	201, 219, 247
RN	RORIDIN A	532	2.3	18	135, 153, 401
	RORIDIN D	530	2.3	24	135, 153, 401
	RORIDIN E	514	2.3	19	385, 403, 359
BN	RORIDIN H	512	2.3	18	109, 137, 125
	B4	562	2.3	19	135, 153, 417
	B5	562	2.3	20	153, 385, 417

Table 4. Screening of Baccharis Plants for Roridins and Baccharinoids

	RORIDIN A			RORIDIN D			RORIDIN E			RORIDIN H			B4			B5		
SAMPLE	249	231	137	249	231	137	361	231	267	137	231	359	247	137	157	247	201	219
STANDARD	100	33.4	11.8	100	57.8	63.6	100	67.6	5.0	100	74.7	48.7	100	94.7	6.4	100	15.2	9.0
PLANT 1	100	40.9	26.9 •	43.6	100	81.4	100	7.1	3.8 d	100	21.4	—	—	100	—	13.9	27.3	100
PLANT 3	100	33.5	17.7 •	46.5	64.0	100	100	21.9	4.0 d	100	23.7	—	—	100	—	100	70.0	70.6 d
PLANT 4	100	29.6	16.8 •	72.2	70.3	100	100	23.8	23.0 d	100	0.6	—	100	22.4	8.6	100	23.0	16.1 •
PLANT 6	100	26.4	19.7 •	56.9	66.2	100	100	16.6	4.1 d	100	63.2	—	—	100	3.5	100	56.2	77.3
PLANT 10	62.3	50.5	100	100	10.7	56.4 d	100	39.5	42.9 d	100	0.1	—	100	14.0	11.4	100	26.0	7.2 •
PLANT 13	100	35.7	22.9 •	66.9	77.2	100	100	21.9	16.1 d	100	62.2	15.1	22.6	100	—	13.6	60.5	100
PLANT 15	100	29.6	16.9 •	72.6	70.6	100	100	24.2	23.1 •	69.7	100	—	56.0	100	—	9.6	61.6	100

• PRESENCE
d DOUBTFUL

The criteria for identifying macrocyclic trichothecenes in samples were identified according to the appropriate, most abundant ion and the relative abundances and similarities of the other two daughter ions (Table 4). The sample data listed in Table 4 and the measured data of the samples were analyzed according to these criteria. In Table 4, definitively identified compounds are indicated by an asterisk, whereas doubtful identities are marked as "d." Roridin A was identified in all the plant samples except 10. Baccharinoid 5 was detected in plant samples 4 and 10, and Roridin E was found in plant sample 15. However, the presence of Roridin D in plant sample 10 and Roridin E in all but plant sample 15 was doubtful. Baccharinoid 5 in plant sample 3 remained uncertain based on observed ratios.

Hence, we investigated the possibility of combining negative CAD ions with positive ones to detect more conclusively, even during the initial screening process, the presence of roridins and baccharinoids. The measurement of intensities of the negatively charged daughter ions, as specified in experiments BN and RN (Table 3), was also attempted. The intensities of the ions and their ratios for baccharinols could not be reproduced over a period of a few hours even with standard. This fact contradicts the observed results when methane was used as the CI reagent gas.[15,16] The ion intensities of the negative daughter ions varied more dramatically during the analysis of samples, and the ion volume (source) needed cleaning after a few experiments. Similar observations were also made while monitoring the parent ions of m/z 401, m/z 359, and m/z 417. Hence, the attempt to use negative CAD ions during the screening was not pursued further.

Under the same conditions, the positive and negative daughter spectra of KVA (the intended internal standard) were recorded, and the fragmentation mode was similar to that of the analytes. The positive CAD spectrum of KVA is shown in Figure 9. The alternate mode of screening these molecules is shown in Table 5. To check its behavior on the molecules and its possible use as the internal standard, the KVA was also introduced in experiments X1 and X2.

Quadrupole 1 (Q1) and quadrupole 3 (Q3) were tuned specifically to obtain the parent spectra in the positive mode using perfluorotributylamine (PFTBA) for calibration and tuning to obtain the parent spectrum of m/z 219. The collision gas pressure and the collision energies were maintained at 1 mTorr and 14eV, respectively. The standards for roridins and baccharinoids (25 ng) were analyzed separately under the experiments (X1 and X2) outlined in Table 5. The specified conditions were adequate, and the ratios of the characteristic daughter ions for all compounds were calculated. One microliter of the sample solutions along with 25 ng of KVA was also analyzed under experiments X1 and X2. The daughter ion intensities and their ratios were measured and compared with the values observed for the standard. In addition to the identification criteria specified earlier, the information from the parent spectral data was used to detect the diesters in the samples.

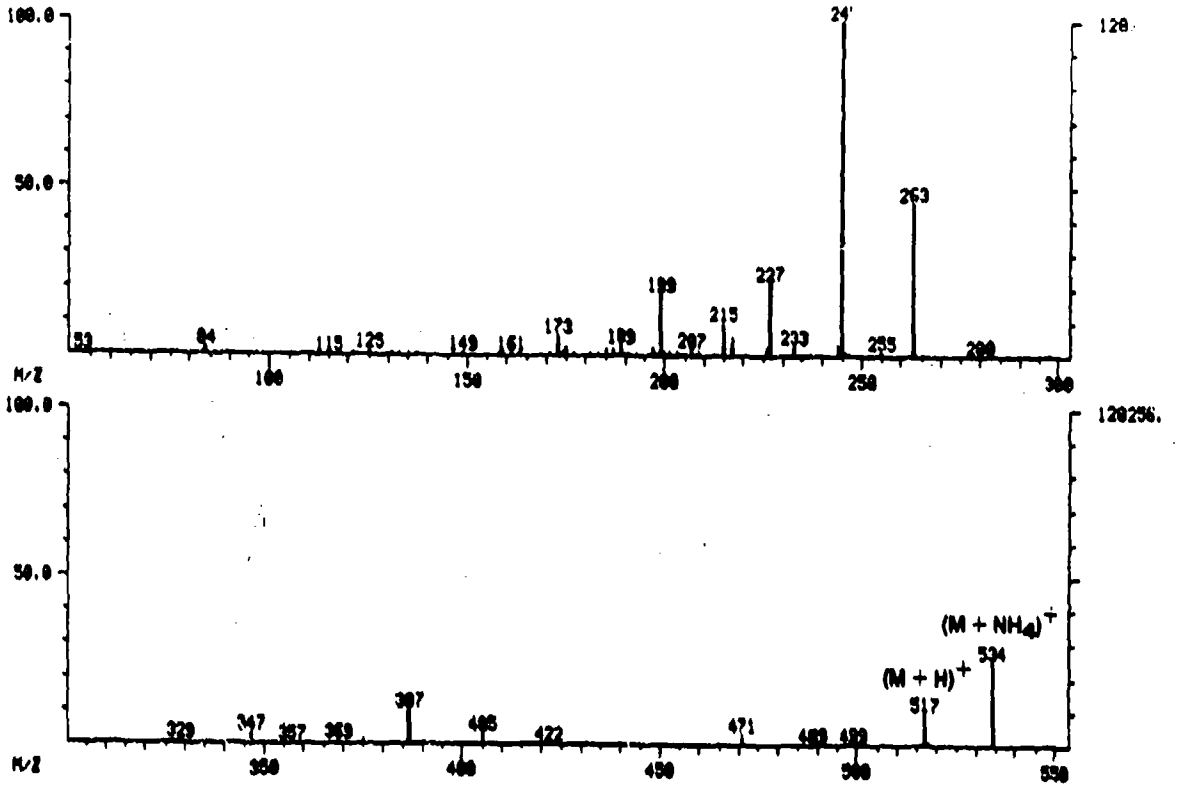


Figure 9. Daughter Spectrum of 8-Ketoverrucarin A - Ammonium Adduct (m/z 534)

Table 5. Alternate Screening Conditions*

EXPERIMENT	MASS SETTING (m/z) Q_1	MASS SETTING (m/z) Q_3	TIME OF MONITORING (SECONDS)	COMPOUND
X1	550	137, 231, 249	0.09	RORIDIN A
	548	137, 231, 249	0.09	RORIDIN D
	532	231, 267, 361	0.09	RORIDIN E
	530	137, 231, 359	0.09	RORIDIN H
	532	199, 245, 263	0.09	KVA (Internal Standard)
	500-600	249	0.08	RORIDIN A, D, H
	500-600	361	0.08	RORIDIN E
X2	580	137, 157, 247	0.09	B4
	580	201, 219, 247	0.09	B5
	534	199, 245, 263	0.09	KVA
	500-600	247	0.08	B4 and B5

* Parent spectral tuning. CE, -14.0 ev; Collision gas pressure: 1.0 mtorr

The compounds identified by experiments RB and RS (Table 4) were also detected and confirmed under these conditions (Table 5). In addition, the presence of Roridin E was also confirmed in plant samples 1, 3, 10, 13, and 15. Its presence was not found in samples 4 and 6. The absence of Roridin D and B5 in samples 10 and 3, respectively, was also deduced. These results were confirmed by obtaining full-scan daughter spectra of all the samples for all the compounds detected. These results agreed with the observations of Jarvis and others who have isolated, purified, and characterized these trichothecenes from several kilogram quantities of the corresponding plant samples (Personal communication with B.B. Jarvis, University of Maryland). Some of the full-scan daughter spectra of the samples are shown in Figures 9-12. The relative abundances (RA) in three characteristic daughter ions of KVA were measured from all sample runs. The RA of m/z 199 (14.5 $\pm$ 2.3) and m/z 263 (47.6 $\pm$ 5.2) remained reasonably constant even in sample matrices. This series of experiments also showed that the optimum instrumental and CAD conditions for obtaining the parent spectra were adequate for simultaneously screening compounds by parent and daughter spectral modes.

The following series of experiments were designed and conducted to determine the optimum conditions for quantifying the detected analytes. Using a collision energy of -14 eV, a mixture containing 100 pg of roridins, B4, B5, and KVA was analyzed at 1 mTorr, 1.5 mTorr, and 2.3 mTorr, respectively. Three daughter ions for each compound specified (Table 4) were monitored, and their ion intensities were measured. In most instances, the optimum CAD conditions for the abundant production of daughter ions (base peak) were -14 eV and 2.3 mTorr. Thus, using tuning conditions adequate for obtaining daughter spectra, all quantification measurements were made under these same conditions.

Along with 100 pg of KVA, 5-30,000 pg of roridins and baccharinoids were analyzed under the conditions specified in Table 6. The ion intensities of the most abundant ion of all those analyzed and the corresponding relative amounts of the ion intensities were measured with respect to the internal standard (m/z 245). A compound's relative ion intensities were plotted against their relative amounts, and a linear relationship over 3 orders of magnitude was observed between the intensities and amounts. Along with their linear regression constants, the linear response ranges of the roridins and baccharinoids are shown in Table 7. The response factors (an average value of 10 measurements or more) of all the analytes with respect to KVA were also calculated and are listed in the same table. The standard deviation values for the response factors and the correlation coefficients indicated that quantifying these macrocyclic trichothecenes could be done with excellent precision. This information and the consistent RA of the daughter ions observed in sample matrices indicated that these analytes could be accurately quantified in samples by introducing correction factors if or when required.

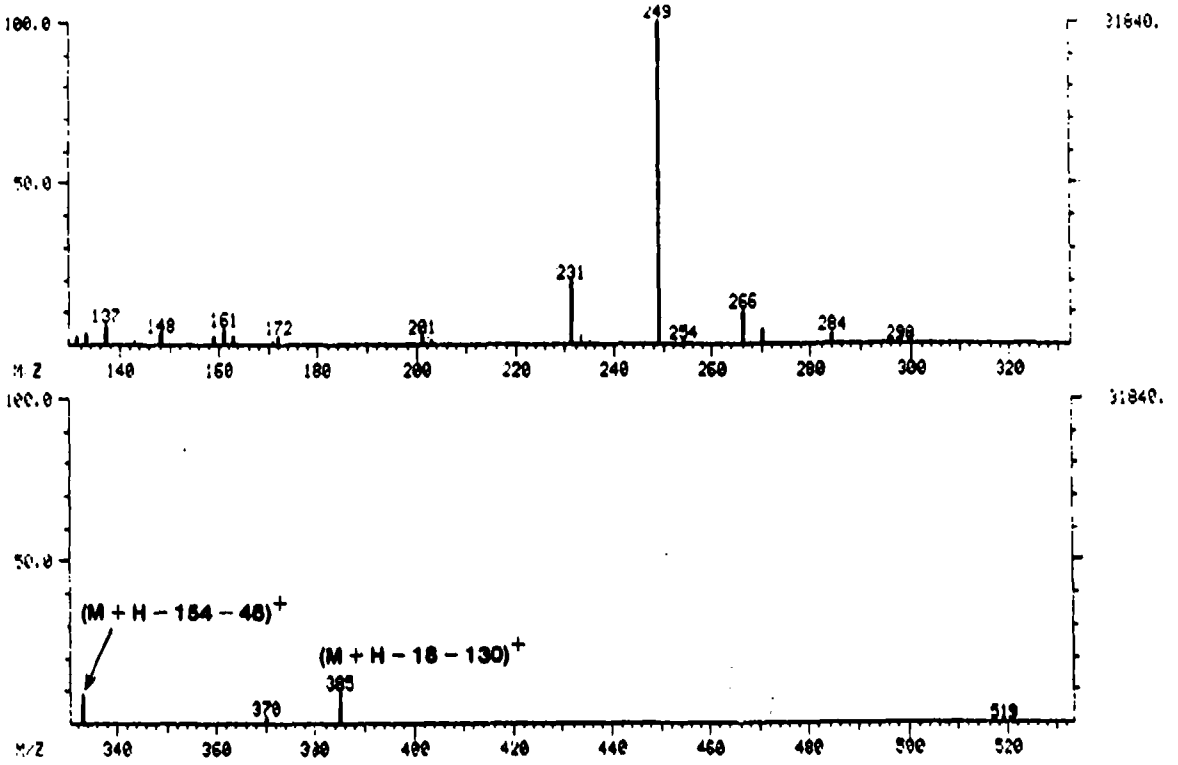


Figure 10. Daughter Spectrum of m/z 550 in Baccharis Coridifolia Plant Sample 15

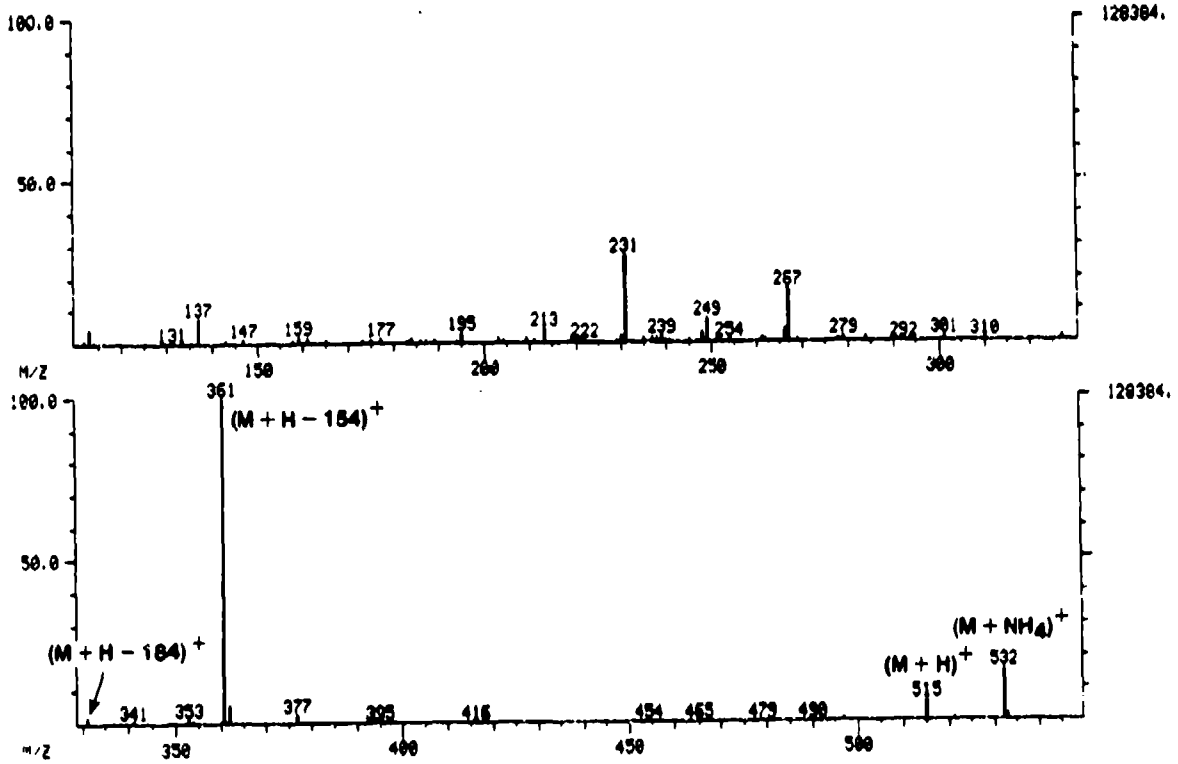


Figure 11. Daughter Spectrum of m/z 532 in Baccharis Coridifolia Plant Sample 15

Table 6. Quantification Conditions

COMPOUND	MASS/CHARGE (m/z) Q_1	MASS/CHARGE (m/z) Q_3	MONITORING TIME (SECONDS)
RORIDIN A	550.3	249.2	0.06
RORIDIN D	548.3	249.2	0.06
RORIDIN E	532.3	361.2	0.06
RORIDIN H	530.3	137.1	0.06
B4, B5	580.3	247.2	0.06
KVA	534.3	246.2	0.06

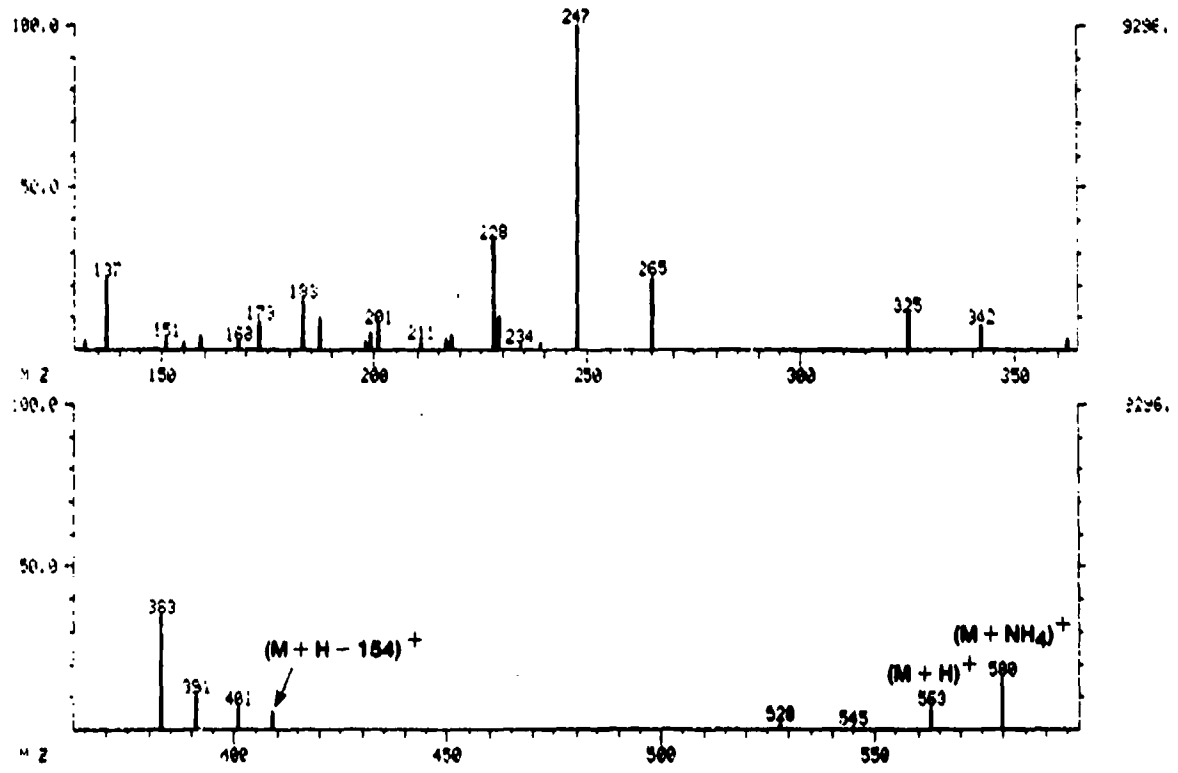


Figure 12. Daughter Spectrum of m/z 580 in Baccharis Megapotamica Plant Sample 10

Table 7. Calibration Data

COMPOUND	MASS/CHARGE Q1	MASS/CHARGE Q3	RESPONSE FACTOR[a]	LINEAR RESPONSE RANGE(Pg)	CORRELATION COEFFICIENT	SLOPE	INTERCEPT
Roridin A	550	249	0.15 ±0.02	5–5000	0.963	0.110	0.147
Roridin D	548	249	0.17 ±0.03	5–5000	0.998	0.127	0.054
Roridin E	532	361	0.04 ±0.01	5–10000	0.985	0.048	−0.088
Roridin H	530	137	0.16 ±0.02	5–10000	0.990	0.160	−0.010
Baccharinoids	580	247	0.02 ±0.003	10–20000	0.997	0.023	−0.07
KVA (Internal Standard)	534	245	1.000	-	-	-	-

[a]value from 10 measurements or more.
Q1 =Quadrupole 1
Q3 =Quadrupole 3

The detected compounds in plant samples 10, 13, and 15 were quantified by analyzing 1 µl of the extract in methanol and 100 pg of KVA by the proccdure indicated in Table 6. To calculate their relative amounts and the amount of the analytes in the analyzed portion of the sample according to the internal standard, the measured relative ion intensities were extrapolated into their corresponding calibration curves. When the measured quantities of the analytes exceeded the linear response range, the sample was diluted further and analyzed once more; the quantification results of the additional analysis are shown in Table 7. Six spiked solutions of plant extract 10 containing 50 pg to 5 ng/µl of B5 were prepared and analyzed to quantify by the standard addition method, the amount of the baccharinoid present in the sample. The measured ion intensity ratios were plotted against the amount of the spiked standard, and the interception of the plot indicated the ion intensity ratio of B5 in the sample. The measured quantities of the compound (standard addition method) in the analyzed amount and the total amount of the sample are listed in parenthesis in Table 8. When the direct analysis method was used to quantify B5 in sample 10, the resulting value agreed with the value obtained by the standard addition procedure. Similar results were observed for sample 15 when it was spiked with Roridins A and E. The measured concentrations of Roridins A and E (sample 15) by both processes are shown in Table 8. The matrix effect seems insignificant during these quantification procedures. KVA was adequate as an internal standard for quantifying roridins and baccharinoids by the PICI-MS/MS technique as well. Earlier investigations found KVA to be adequate also for quantifying satratoxins by the NICI-MS/MS procedure.[16]

Table 8. Quantification of Baccharis Plant Samples

EXTRACT SAMPLE #	ANALYZED FRACTION	IDENTIFIED COMPOUND	AMOUNT ANALYZED* (Ng)	AMOUNT TOTAL SAMPLE (µg)
10	1/10,000	RORIDIN E	0.18	1.8
		B5	38.7	387
	1/100,000	B5	7.8 (8.8)	780 (880)
13	1/1,000	RORIDIN A	28.9	28.9
		RORIDIN E	66.5	66.5
	1/10,000	RORIDIN A	2.6	26
		RORIDIN E	6.3	63
15	1/10,000	RORIDIN A	7.2	72
		RORIDIN E	11.4	114
	1/100,000	RORIDIN A	0.55 (0.50)	55 (50)
		RORIDIN E	1.0 (0.95)	100 (95)

*Values mentioned in the parentheses were obtained by standard addition method.

Several experiments were performed to measure the minimum detectable levels of roridins and baccharinoids. Initially, three positive daughter ions of a compound were monitored with an individual standard (Table 5). All ions were observed with the specific RA with signal-to-noise (S/N) ratios of at least 10:1. The minimum detectable levels determined for roridins and baccharinoids were 10 and 20 pg, respectively, and the values for these trichothecenes are listed in Table 9 under individual (IN) experiment. In experiment R1, three daughter ions of KVA were sequentially monitored along with Roridins A, D, and E, and in R2, three CAD ions of Roridin H and B4 and B5 were sequentially observed along with KVA. Three daughter ions of all the analytes and KVA were monitored sequentially in experiment RB. The three daughter ions of the roridins and KVA and three parent spectra of m/z 249 and m/z 361 were obtained alternatively in experiment X1. Similarly, for daughter ions of baccharinols and KVA and parent spectra of m/z 247 (baccharinoid), experiment X2 (Table 6) was used. In experiment QA, the most abundant daughter ion of all the analytes and internal standard (KVA) was monitored sequentially. The minimum amounts of all compounds detected under all the above specified conditions are listed in Table 9.

Table 9. Minimum Detectable Levels (Pg)

EXPERIMENT	RORIDIN A	RORIDIN D	RORIDIN E	RORIDIN H	B4	B5	KVA
IN*	10	10	10	10	20	20	10
R1	50	50	75	—	—	—	50
R2	—	—	—	50	100	10L	50
RB	100	100	100	100	150	150	100
X1	150	150	150	150	—	—	100
X2	—	—	—	—	100	100	100
QA	50	50	50	50	50	50	50

*IN - Experiment to monitor 3 daughter ions of one compound

4. CONCLUSIONS

The roridins and two baccharinoids could be identified and accurately analyzed by the PICI-MS/MS technique. Ammonia and argon were the CI reagent and collision gases, respectively. We found that monitoring three characteristic positive daughter ions of each compound and the parent ion of the corresponding most abundant daughter ion will result in the unambiguous detection of the analytes in a single screening step. The detected analytes could be quantified with excellent accuracy using KVA as the internal standard. All the developed experimental procedures were applicable for accurately analyzing real world samples. The minimum detectable quantities for all compounds under various adopted experimental conditions range from 10-150 pg.

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